Challenges in the Identification of New Thermolabile Psychoactive Substances: the 25I-NBOH case.

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Highlights

1. Thermolabile NPS can generate artefacts in the traditional GC-MS analysis
2. When analysed by GC, 25I-NBOH fragments into 2C-I and an ortho-phenolic benzyl ether (o-PBE)
3. No method adjustments can refrain 25I-NBOH thermo degradation on GC analysis
4. DSC and TGA analysis clarify 25I-NBOH´s thermal instability
5. Derivatization can overcome 25I-NBOH degradation in the GC-MS

Abstract

The continuous emergence of NPS over the last years poses a series of novel challenges for forensic analysts. Most of those new compounds are synthesized with minimal chemical modifications to the structure of already known chemicals in order to avoid regulations. Some of these new compounds may undergo chemical changes during analysis leading to misidentification and detrimental legal consequences. GC–MS is one of the most widely used analytical techniques employed by forensic laboratories all over the world for drug analysis. Nevertheless, thermolabile NPS, such as 25I-NBOH can generate artefacts in the traditional GC-MS analysis. In this paper, we describe the fragmentation mechanism of the 25I-NBOH into a major peak corresponding to 2C-I and a minor one corresponding to the associated ortho-phenolic benzyl ether (o-PBE), which exact identity is directly linked with the solvent used for the analysis. Also, a series of method adjustments is displayed, encompassing variation on the injector temperature, split ratio and flow ratio, although with no success to prevent 25I-NBOH thermo degradation in the GC injector. Furthermore, differential scanning calorimetry and thermogravimetric analysis demonstrated that 25I-NBOH´s thermal stability is due to a smaller temperature window between fusion and decomposition points. Finally, we perform derivatization experiments and demonstrate how to overcome 25I-NBOH degradation in the GC/MS analysis.

Key words 25I-NBOH, NPS, thermal degradation, derivatization

Introduction

In the last decade, novel psychoactive substances (NPS) have transformed the global synthetic drug market [1]. Recent advances in organic chemistry have allowed the low cost and easy to replicate synthesis of hundreds of different substances with the desired chemical functionality as to emulate target psychoactive effects in the human body. As a consequence an unprecedented proliferation of NPS, in terms of both quantity and diversity has been witnessed [2]. Moreover, trends on the synthetic drug market quickly evolve each year complicating the challenges to monitor, understand and control of synthetic drugs and their chemicals precursors [3].

One important category of NPS are thermolabile compounds, which are not prone to degrade under typical gas chromatography injection conditions due to the occurrence of strong intramolecular hydrogen bonds. This type of substance will experience thermal degradation and it is not likely to be identified by routine gas chromatographic methods. Among them, the substituted psychedelic phenethylamine 25I-NBOH has become a benchmark case right after NBOMes have been scheduled [4].

An understanding of the thermal behavior of 25I-NBOH is crucial to determine whether degradation can be avoided in GC analysis. Differential scanning calorimetry (DSC) is frequently the preferred thermal analytical technique used because of its ability to provide detailed information regarding both, physical and energetic properties of a substance [5]. This technique provides quantitative information about exothermic, endothermic and heat capacity changes as a function of temperature and determines whether there are underlying slow degradation or decomposition processes occurring [6]. In this context Thermogavimetric Analisys (TGA) also delivery critical information by measuring changes in the physico-chemical properties at elevated temperature as a function of increasing temperature. Therefore essential data regarding stability, decomposition and compound structure can be clarified using TGA [7]. Both methodologies can provide key information to comprehend the degradation process on 25I-NBOH compounds and be a useful tool to drive further actions in an attempt to prevent degradation.

Due to thermo instability, GC analyses of 25I-NBOH will not be able to unequivocally establish if all the 2C-I present in the chromatogram is due to 25I-NBOH thermal degradation alone or whether 2C-I was originally present in the mixture. This fact implies a serious legal problem as 25I-NBOH is not a scheduled drug in many countries whereas 2C-I is [8].

Although other analytical techniques such as differential pulse voltammetry (DPV), near-infrared spectroscopy (NIR) and easy ambient sonic-spray mass spectrometry imaging (EASI-IMS) can successfully overcome this problem [9–11], GC-MS is the most widely used technique for identification/confirmation of drugs by forensic experts. As an example, approximately 90% of forensic laboratories that participate in the International Collaborative exercise proposed by UNODC in 2018 utilized GC-MS as a reference method for drug analyses [12].

Consequently, the development of a improved GC method for the correct identification of 25I-NBOH is of high relevance for forensic analysts all over the world. In this paper, we explore the fragmentation mechanism of the 25I-NBOH, present our efforts to avoid its thermo degradation

in the GC injector, display thermal analysis results and suggest a solution to overcome the presented problem.

Material and Method

Study samples

25I-NBOMe and 25I-NBOH certified standard was purchased from Cayman Chemical (Ann Arbor, MI, USA). 2C-I certified standard was kindly donated by United Nations Office on Drugs and Crime (UNODC). Blotter papers containing 25I-NBOH were donated by Brazilian Federal Police. Methanol, N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA), acetic anhydride and pyridine were purchased from Merck KGaA (Darmstadt, Germany).

Analysis of thermal behavior of 25I-NBOH using DSC and TGA

Differential scanning calorimetry (DSC) of the 25I-NBOH was carried out using a differential scanning calorimeter DSC822e from Mettler-Toledo. For the analysis 2.53mg of 25I-NBOH and about 2.33mg of 25I-NBOMe were used. The calorimeter was programmed for a heating ramp between 30°C and 500°C with a rate of 10°C min^{-1}. Measurements were performed under a Nitrogen (N2) atmosphere with a constant flux of 80 mL min^{-1}.

Thermogravimetric analysis was conducted using a TGA/SDTA851e module (Mettler-Toledo, Columbus, OH, EUA). For the analysis 2.47 mg of 25I-NBOH and 2.22 mg of 25I-NBOMe were used. A heating rate of 20°C min^{-1} was programmed in the temperature range of 30°C to 800°C with a nitrogen (N_2) gas flow rate of 50 mL min^{-1}.

Qualitative analysis of 25I-NBOH by GC-MS

GC-MS analyses were performed on an Agilent 7890A gas chromatograph connected to a 5975C mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The system was controlled by Agilent Chemstation GC-MS Software version E 02.02.1431 (Agilent Technologies, Santa Clara, CA, USA). For the GC-MS analysis an Agilent J&W HP-1MS fused silica capillary column (30m x 0.25 mm x 0.25 μm) was used. Sample injection volume was 1 μL with a 25:1 split ratio. Helium was used as carrier gas, with a constant flow rate of 1 mL min^{-1}. Injector temperature was set to 280°C. The oven program started at 150°C with a hold for 1.5 min, ramped up at 30°C min^{-1} to reach 250°C with a hold for 1 min, and then ramped up at 50 °C min^{-1} to 300°C with a hold for 3 min. The transfer line temperature was set at 300°C. The solvent delay was set to 1.5 min. Mass scan range was *m/z* 35–550.

Derivatization procedure of 25I-NBOH

Three derivatizing agents were tested: N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) and acetic anhydride. For each one, five blotter paper micro stamps originated from Brazilian Federal Police seizures were immersed into 1.5 mL of methanol and extracted in an ultrasound bath for 30 min. The extract was then dried to a powder in a rotary evaporator at room temperature. In the case of MSTFA and BSTFA, 150 μL were added in the dry extract, vortexed for 10s. In the case of acetic anhydride, 100 μL of the

derivatization reagent and 100 µL of pyridine were added the dried extract before vortexing. In all cases, the resulting solutions were kept in a vial heater at 70°C for 1h. 1 µL of the reconstituted solutions, without any additional solvent, was injected into the GC-MS.

Results and Discussion

25I-NBOH in-port degradation and proposed fragmentation of *o*-PBEs

As previously reported [8], 25I-NBOH undergoes degradation during GC analyses. When injected in the presence of an alcohol, two peaks can be observed: a major one corresponding to 2C-I, and a minor one corresponding to the associated ortho-phenolic benzyl ether (*o*-PBE). The exact identity of the *o*-PBE observed is directly linked with the solvent used for the analysis. We propose here the degradation mechanism and how it affects the resulting mass spectra of the different *o*-PBEs created.

When introduced in the injector, 25I-NBOH can be considered as a zwitterion solvated by excess alcohol in the gas phase. Under high temperature conditions (280°C) and relatively high pressure in the liner environment, the alcohol acts as a nucleophilic group and the 2C-I as a leaving group. The electron-rich oxygen from the alcohol replaces the 2C-I group to form an ether. As a zwitterion, the natural tendency of 25I-NBOH to undergo an intra-molecular six-membered proton transfer, leading to a positive nitrogen and a negative oxygen on its phenolic moiety, enables the capturing of the hydrogen from the alcohol's hydroxyl and "facilitates" the nucleophilic substitution (Figure 1 (a)). This interpretation is supported by mass spectra obtained by Neto [8] where 25I-NBOH was solvated in tetradeuterated methanol.

Indeed, while the 2C-I fragmentation mechanism is classically based upon a series of cleaves, the *o*-PBEs presents a more complex picture (Figure 2). When using methanol, ethanol, and *n*-propanol as the solvent, molecular ions $[M]^+$ are observed (respectively *m/z* 138, 152, and 166) as well as a series of identical daughter ions (*m/z* 121, 106, 78, and 51).

Two mechanical pathways, each starting with an α-cleavage on one side of the ether, allows explaining the presence of these ions (Figure 1 (b)). Pathway 1 considers the α-cleavage of the ether moiety. The positively charged intermediate stabilizes its charge in the phenyl ring through tautomeric interconversions, followed by a hydrogen abstraction. It leads to the formation of oxo-tropylium ions (*m/z* 106) via aromatic rearrangement, followed by the α-cleavage of the carbonyl group producing stable phenylium ions (*m/z* 78), and subsequent loss of acetylene ($-C_2H_2$) creating cyclobutandienylium ions (*m/z* 51) [13]. Such patterns have been previously reported for tropones and aromatic systems [14].

Pathway 2 considers the α-cleavage of the alkyl tail of the ether. Two subsequent abstractions of hydrogen result in the production of [(2-hydroxyphenyl)methylidyne]oxonium ions (*m/z* 121). The particularity of this ion is that it is the only daughter ion retaining the hydrogen originally provided by the solvent to the *o*-PBE. Indeed, this is why the mass spectrum of the o-PBE obtained with tetradeuterated methanol [8] shows a *m/z* 122 ion, corresponding to the *m/z* 121 ion when using non-deuterated solvents. This is strong evidence supporting the degradation mechanism proposed in Figure 1 (a).

Finally, when using i-propanol as the solvent, the *o*-PBE mass spectrum presented some discrepancies compared to its linear counterpart (Figure 2). During its fragmentation, the 2-[(propan-2-yloxy)methyl]phenol mechanically undergoes pathway 1 with the production of *m/z* 51, 78 and 106. However, due to the absence of *m/z* 121 and the presence of *m/z* 43 and 124 instead, it is likely that instead of pathway 2, these ions undergo an onium reaction where the alkyl linked to the charged heteroatom. In this case the oxonium ion is cleaved with a transfer of a hydrogen from the alkyl substituent to the heteroatom leading to propylium ions (*m/z* 43) and 2-(hydroxymethyl)phenol ions (*m/z* 124) (Figure 1 (c)). Proposing the exact reason for this mechanical change in the fragmentation would be speculative because the precise origin of the hydrogen that is transferred is not known in such type of reaction, however they have been observed in ethers decomposition sequences [13].

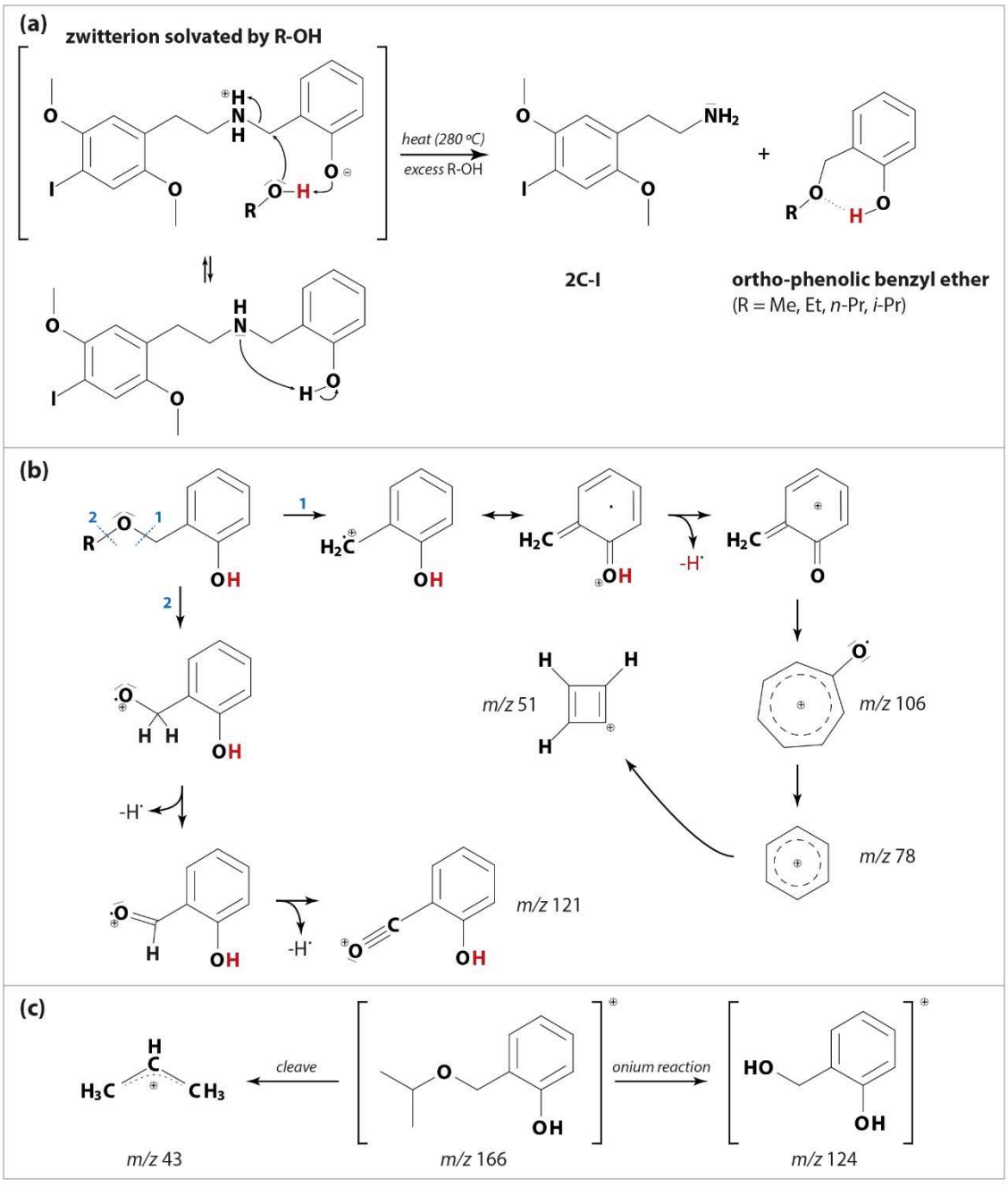

Fig 1 (a)Thermal degradation of 25I-NBOH mechanism and production of 2C-I and *o*-PBEs; (b) *o*-PBEs EI^+ fragmentation pathways (R = Me, Et, *n*-Pr); (c) 2-[(propan-2-yloxy)methyl]phenol EI^+ fragmentation (*o*-PBE created with *i*-propanol as the solvent)

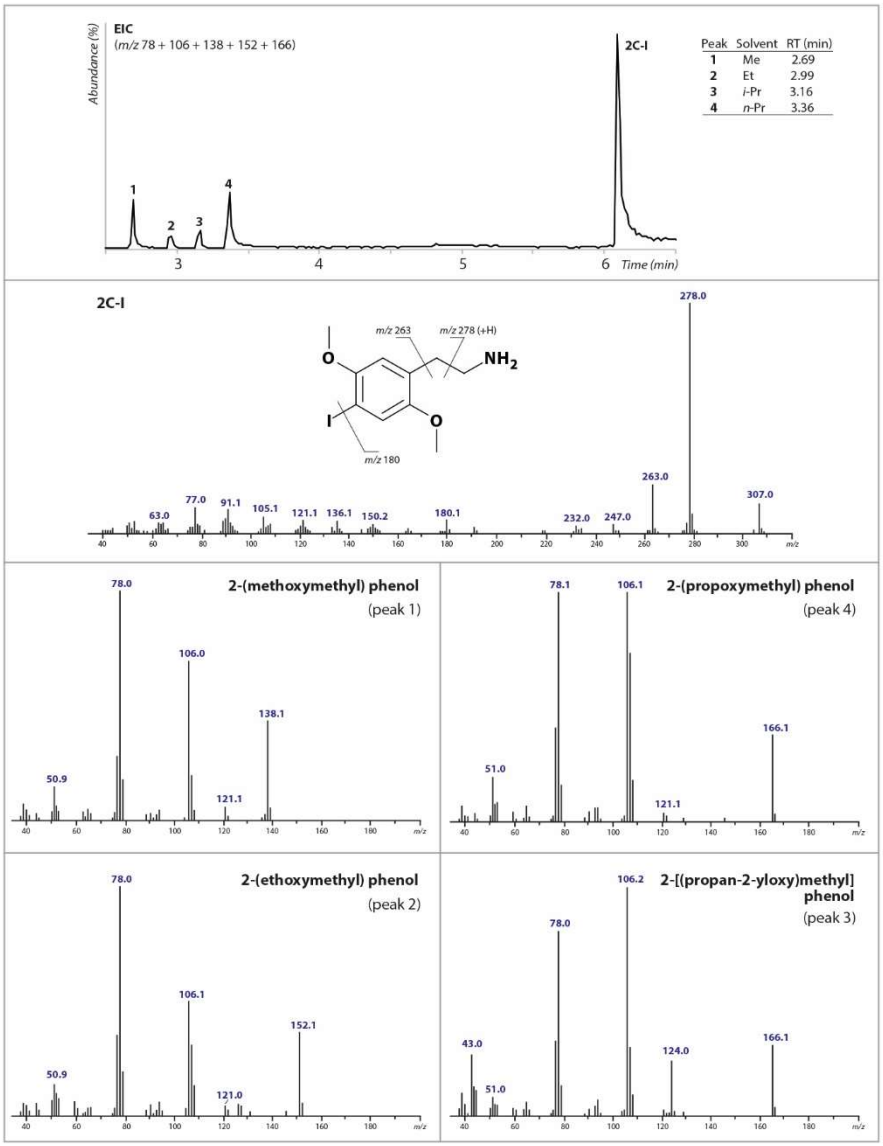

Fig 2 EI^+ extracted ion chromatogram (EIC) (*m/z* 78, 106, 138, 152, and 166) obtained when analysing 25I-NBOH with a mix solvent (Me, Et, *n*-Pr, *i*-Pr) and corresponding respective mass spectra of the 2C-I and the *o*-PBEs created.

Method Adjustments

In order to avoid the 25I-NBOH fragmentation into 2-(methoxymethyl)phenol (peak 1) and 2C-I (peak 2), several methods' modifications were experimented. Adjustments in the injector temperature were the first parameter optimized. Assuming that a thermal degradation occurred in the injector (due to sharp peak shape), temperature was lowered from 280°C to 170°C. The lower the injector temperature, the more difficult it is for the compound to vaporize and a lower signal for peak 2 was achieved, confirming the results previously described by Coelho Neto *el at*

[8]. The 25I-NBOH molecular peak was not observed in any chromatogram. The peaks behavior associated to injector temperature changes can be observed in Figure 3.

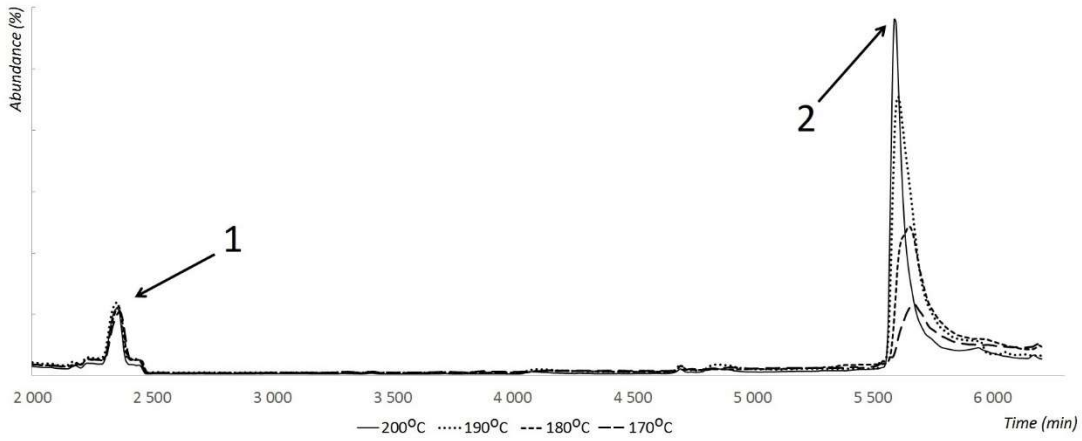

Fig 3 Injector temperature influence on peaks 1 and 2 on a 25I-NBOH GC analysis.

Secondly, split ratio was adapted to maximize the possibility of occurrence of 25I-NBOH molecular ion in the chromatography results. Split (50:1, 25:1, 10:1) and splitless injections were tested. The best abundance detected on peaks 1 and 2 was using split ratio 10:1, nonetheless no molecular peak was recognized on those runs. Those results are in agreement with the ones previous reported [8].

In an attempt to reduce elution temperature and reduce the prospect thermal degradation experiments with different flows rate (0.6 to 1.5mL/min) were conducted. For the smaller fragment (peak 1), flow rate of 0.8 mL/min culminate in better peak abundance. Although for peak 2, flow rate of 1.2 mL/min presented better results.

Thermal Analysis

Despite all the efforts and experiments carried out, the 25I-NBOH molecular ion has not been detected as a result of the mere change in the chromatographic conditions. Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) analysis were carried out with the aim of understanding the thermal behavior of 25I-NBOH in comparison with its less polar counterpart 25I-NBOMe.

Thermal curves, as determined by DSC, are shown in Figure 4(a) For both compounds, 25I-NBOMe and 25I-NBOH. The DSC curves displayed one major endothermic region corresponding to fusion peaks (167^{o}C - 25I- NBOMe; 203^{o}C - 25I-NBOH) and one exothermic region corresponding to a common decomposition peak (265^{o}C - 25I-NBOMe and 25I-NBOH). The shapes of 25I-NBOMe and 25I-NBOH decomposition peaks are very distinct with 25I-NBOH peak more asymmetric with a prominent fronting pattern. Comparing both compounds, a difference of almost 40^{o}C between fusion points was registered. This difference demonstrates that 25I-NBOH has a smaller temperature window between fusion and decomposition directly reflecting on its thermal stability. The 25I-NBOH DSC thermogram shows that as soon as the compound reaches fusion it will start decomposing supporting the fact that only degradation products may be seen in GC-MS analysis.

TGA thermograms, shown on Figure 4(b), corroborate the occurrence for both compounds of a common thermal decomposition at approximately 265°C, which is evidenced by a significant sample mass loss.

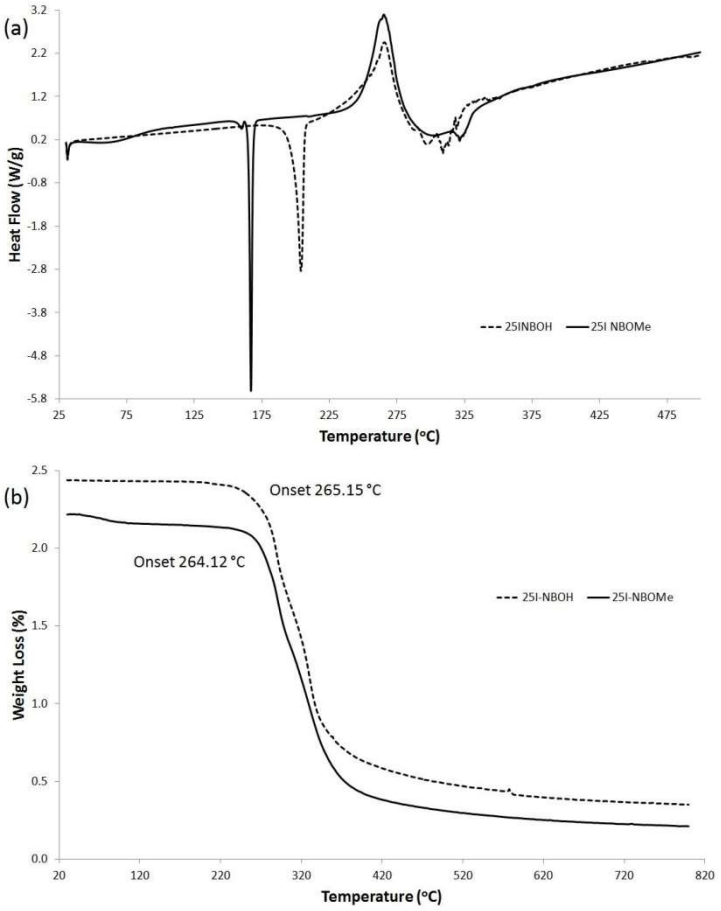

Fig 4 Thermal analysis for 25I-NBOH and 25I-NBOMe. (a) Differential Scanning Calorimetry (DSC) of 25I-NBOH and 25I-NBOMe. (b) Thermogravimetric Analysis (TGA) of 25I-NBOH and 25I-NBOMe.

Derivatization

Thermal analysis results indicated that no GC method adjustment would be capable of unequivocally identifying 25I-NBOH. Consequently, derivatization was investigated as the next approach to overcome 25I-NBOH thermal degradation. A series of experiments using three different derivatization agents (MSTFA, BSTFA, and acetic anhydride) was performed.

MSTFA silylation reaction generated two derivatives: mono-TMS and di-TMS derivatives, which followed the expected order of elution for such species with the mono-TMS version eluting slightly ahead of its di-TMS counterpart (RT = 7.89 min and 7.98 min respectively) (Figure 5). Mono-TMs was produced when MSTFA reacted just with the -OH present in the 25I-NBOH molecule. This fact was confirmed as m/z = 179 is formed by heterolytic cleavage at the amino group resulting in a highly stable tropilic ion with the Si(CH3)3 group attached to the OH in the molecule (Figure 5). Di-TMS is generated when a second derivatization reaction takes place at the –NH site. Distinctly, using BSTFA derivatization only mono-TMS derivative is visualized

(Figure 5). Besides the production of two compounds in the derivatization process, higher chromatographic peak abundances indicate MSTFA as a more effective derivatization reagent than BSTFA.

On the other derivatization experiment, acetic anhydride was used to produce an acylation reaction as it is known to improve the stability of compounds that are thermally labile by inserting protecting groups into the molecule[15]. The acylation promoted the introduction of two acyl groups on both active hydrogens (-OH and -NH) and yielded high intensity di-acylated derivative chromatograms (Figure 5). As in the case of MSTFA the use of acylation indicated the presence of glucose (or any of its stereoisomers) in some of the blotter paper samples with a good match (926 out of 1000) for its pentaacetate form in the NIST 11 mass spectral library. It is possible that sugar has been added on blotter papers with the intent of hiding the alleged bitter taste of 25I-NBOH previously reported in user forums[16–18].

It can be noted that the typical 2C-I peak found in underivatized run (RT 6.16) was not observed with the derivatized run. This is indication that the use of derivatization reagent in excess and lengthy incubation time enabled completion of the derivatization process and that the molecule was “stabilized" through the process, enabling unequivocal identification.

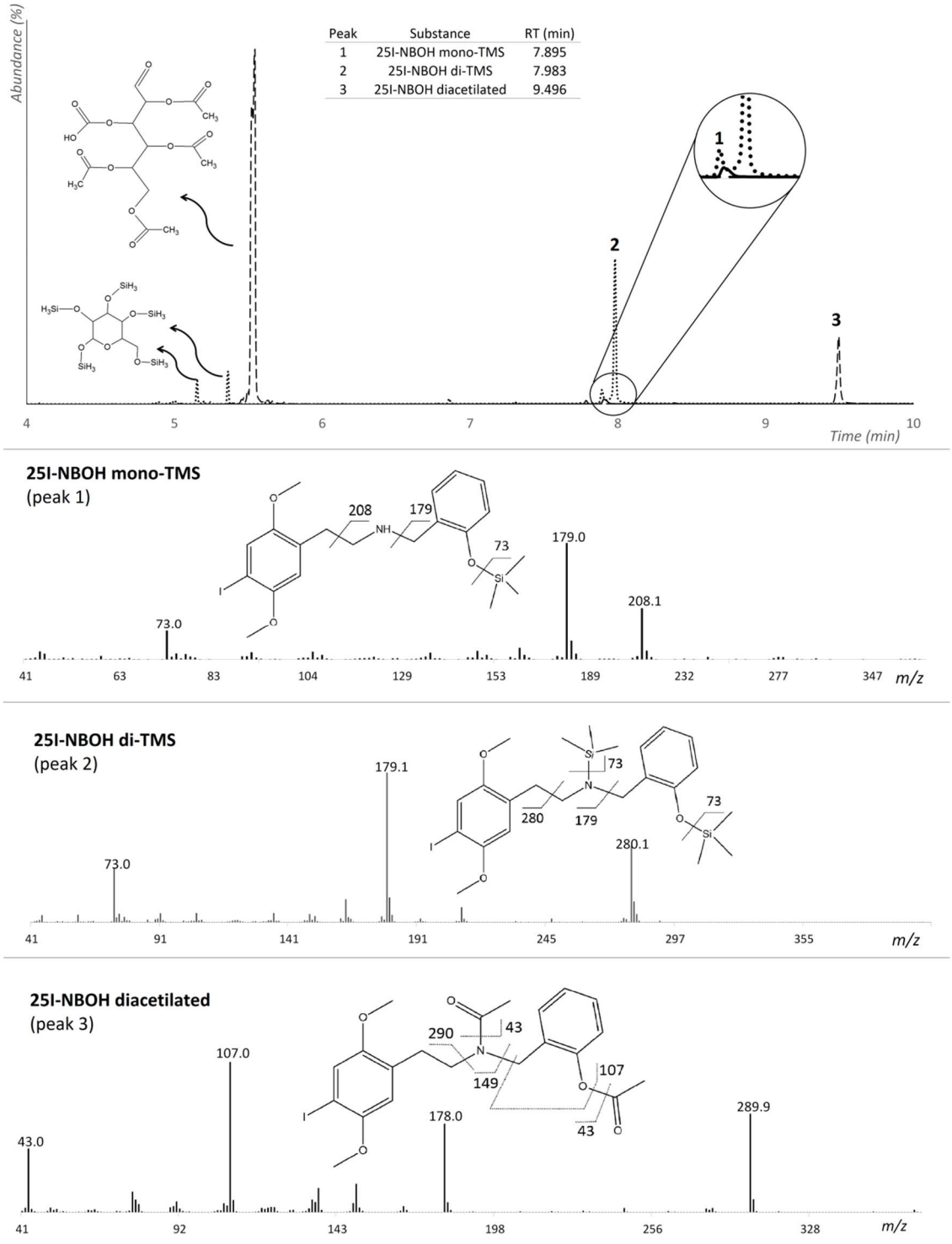

Fig 5 GC-MS profiles and molecular structure obtained after chemical derivatization of 25I-NBOh with MSTFA (·····), BSTFA (——) and acetic anhydride (------).

Conclusion

In this paper, we provided a detailed study of the 25I-NBOH thermal behavior. Degradation and fragmentation pathways were presented and deciphered while DSC and TGA analysis demonstrated that prompt decomposing occurs when the compound reaches fusion point.

Despite all the effort adjusting the GC-MS methods, 25I-NBOH degradation in the injector was unavoidable. However, derivatization enabled the stabilization of the molecule and identification could be successfully made *via* both silylated and acylated derivatives. This indicates how GC-MS can be used to provide correct identification for this class of compounds.

Finally, forensic experts need to be aware of these cases in order to implement non-thermal alternative analytical techniques and chemical derivatization in order to correctly identify the ever-emerging new classes of NPS.

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Compliance with ethical standards

Conflict of interest Authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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